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Research Article

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Model-based kinetic parameters estimation in batch Pullulan fermentation using Jaggery as substrate

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ABSTRACT

The research was focused on validation of unstructured, mathematical, kinetic model data obtained in batch shake-flask studies of Pullulan produced by Aureobasidium pullulans MTCC 2195. Logistic (L), Logistic Incorporated Leudeking-Piret (LILP) and Logistic Incorporated Modified Leudeking-Piret (LIMLP) models were used for kinetic parameters estimation. Very good fit of the data was observed between the experimental data and modelled data in biomass growth, substrate consumption and product formation kinetics. Estimated kinetic parameters like μ_{max}, X_0, X_m, α, β, γ, and η, were compared for initial Jaggery and Sucrose (as substrate) concentrations of 50, 75 and 100 g/L. Furthermore, all these parameters were predicted accurately with reasonable R^2 values.

Keywords: Kinetic model, Pullulan, Jaggery, Sucrose, Validation.

INTRODUCTION

Aureobasidium pullulans, a polymorphic fungus, produces an extracellular and linear polysaccharide called pullulan, which consists of maltotriose subunits interlinked by (1→6)-α-D-glucosidic and α-(1→4)-glycosidic bonds [1, 2]. Due to distinctive physical, chemical and biological properties, pullulan offers excellent applications in food, pharma, cosmetic and packaging industries and environmental clean-up agents [3, 4, 5]. Several researchers varied the strategies for successful production of pullulan includes: effects of carbon substrates and their sources [6, 7, 8, 9, 10, 11]; operational parameters like shear stress, agitation, aeration, DO levels, etc. [12, 13]; medium optimization studies [14], etc.

Mathematical models of fermentation offer knowledge of kinetic and metabolic nature of product and also account biomass as one variable to represent the overall kinetics. Several rate models were successfully proven to estimate the kinetic parameters for growth studies in variety of biopolymers [15, 16, 17, 18, 19]. A very useful unstructured kinetic model for pullulan fermentation was developed for *A.pullulans* growth, limited substrate consumption and pullulan production [20, 21, 22, 23].

The logistic function model developed by Pearl and Reed, 1920 [24], can be applied to *A.pullulans* growth and logistic equation proposed by Mulchandani et al., 1988 [25] was used to calculate the kinetics of batch cultivation of microbial polysaccharide production. These models explain the microbial growth as a function of maximum cell concentration (X_m), maximum specific growth rate and time. Mohammad et al., 1995 [6] developed the use of Leudeking-Piret model for pullulan production and a modified Leudeking-Piret model for sucrose consumption.

Pullulan production was initiated in the late exponential phase, which was more even higher when cells approach stationary phase [3, 26]. In the present investigation, experimental data obtained in shake-flask studies of pullulan fermentation using Jaggery as a new substrate with developed kinetic models were validated.

EXPERIMENTAL SECTION

Microorganism and culture media:
In this study, *Aureobasidium pullulans* MTCC 1991 obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh was procured to produce pullulan from Jaggery as carbon substrate. *A. pullulans* was maintained on potato dextrose agar (PDA) and grown on standard cultivation media with a composition of sucrose: 50.0 g/L; yeast extract: 3.0 g/L; KH_2PO_4: 5.0 g/L; KCl: 0.5 g/L; $MgSO_4 \cdot 7H_2O$: 0.2 g/L; NaCl, 1.0 g/L in 1 liter of distilled water. Jaggery was purchased from local market and sucrose was replaced by jaggery in standard cultivation media and used further in shake flask fermentation studies.

Studies of shake flask fermentations were carried out with standard cultivation medium containing sucrose and also medium containing jaggery with varying concentrations of 50, 75 and 100 g/L. All the preparations were made to 100 ml aliquots in 500 ml Erlenmeyer flasks and sterilized. The sterilized media was incubated on an orbital incubator shaker at 30^0C and 150 rpm for 172 hours, after inoculating with 5% (v/v) inoculum. At regular intervals, samples from shake flasks were drawn and analyzed for dry cell weight of biomass, pullulan and sugar content. All the experiments were carried out in triplicates and the average values of data were used.

Biomass (cell dry weight), Pullulan and Sugar (residual) content estimation:
Estimation of dry cell weight of *A.pullulans* was followed as per Vijayendra et al., 2001 [27] and the residual sugar content in fermentation broth supernatant was measured as per Miller's 1959 [28] method. Produced pullulan was estimated by solvent precipitation, subsequent filtration and drying [27, 29, 30].

Kinetic study model:
The kinetic models for cell growth, substrate consumption and pullulan (as product) synthesis in a batch system was developed by many researchers [6, 20, 21, 22, 23]. Under optimal growth conditions, growth kinetic model of *A. pullulans* (*X*) (as per Malthus's law), in a batch fermentation is given as:

$$\frac{dX}{dt} = \mu_{max} X \left(1 - \frac{X}{X_m}\right) \quad (1)$$

The Logistic (L)- type model equation derived from the integration of above equation results:

$$X(t) = \frac{X_0 e^{\mu_{max} t}}{1 - \frac{X_0}{X_m}(1 - e^{\mu_{max} t})} \quad (2)$$

A plot of $\ln\left(\frac{X_t(X_m - X_0)}{X_0(X_m - X(t))}\right)$ vs *t* will yield maximum specific growth rate of biomass, μ_{max} as slope.

The substrate utilization kinetics in microbial polysaccharide production can be taken from Modified Leudeking-Piret (MLP) equation:

$$-\frac{dS}{dt} = r_S = \gamma\left(\frac{dX}{dt}\right) + \eta X \quad (3)$$

Logistic Incorporated Modified Leudeking-Piret (LIMLP) equation derived from integration of above equation results:

$$S(t) = S_0 - \gamma\left[\frac{X_0 e^{\mu_{max} t}}{1 - \left(\frac{X_0}{X_m}\right)(1 - e^{\mu_{max} t})} - X_0\right] + \frac{\eta X_m}{\mu_{max}} ln\left[1 - \left(\frac{X_0}{X_m}\right)(1 - e^{\mu_{max} t})\right] \quad (4)$$

Non-growth associated constant, η, in above equation can be calculated from stationary phase data (where $\frac{-dS}{dt} = 0$):

$$\eta = \frac{-\left(\frac{dS}{dt}\right)_{stationary\ phase}}{X_{max}} \quad (5)$$

And, a plot of $(S_0 - S(t)) + \frac{\eta X_m}{\mu_{max}} ln\left[1 - \left(\frac{X_0}{X_m}\right)(1 - e^{\mu_{max} t})\right]$ vs $\left[\frac{X_0 e^{\mu_{max} t}}{1 - \left(\frac{X_0}{X_m}\right)(1 - e^{\mu_{max} t})} - X_0\right]$ will yield growth-associated constant, γ as slope.

Product formation kinetics follows Leudeking-Piret equation, as:

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \quad (6)$$

Logistic Incorporated Leudeking-Piret (LILP) equation derived from integration of above equation results:

$$P(t) = P_0 + \alpha \left[\frac{X_0 e^{\mu_{max} t}}{1-\left(\frac{X_0}{X_m}\right)(1-e^{\mu_{max} t})} - X_0 \right] + \frac{\beta X_m}{\mu} ln \left[1 - \left(\frac{X_0}{X_m}\right)(1 - e^{\mu_{max} t}) \right] \quad (7)$$

β, non-growth associated parameter can be determined from stationary phase data (where $\frac{dX}{dt} = 0$):

$$\beta = \frac{\left(\frac{dP}{dt}\right)_{stationary\ phase}}{X_{max}} \quad (8)$$

A plot of $(P_t\text{-}P_0) + \frac{\beta X_m}{\mu} ln \left[1 - \left(\frac{X_0}{X_m}\right)(1 - e^{\mu_{max} t}) \right]$ vs $\left[\frac{X_0 e^{\mu_{max} t}}{1-\left(\frac{X_0}{X_m}\right)(1-e^{\mu_{max} t})} - X_0 \right]$ yields growth-associated parameter, α as slope.

In this study, equations (2), (4) and (7) were used to simulate the experimental data obtained in the shake-flask batch fermentations with initial sucrose and Jaggery concentrations of 50, 75 and 100 g/L. The software Microsoft Excel 2010 was employed to estimate the values of modelled kinetic parameters.

RESULTS AND DISCUSSION

Data, used in this study were taken from average of three shake-flask studies of pullulan fermentation. All models were developed in a manner that they contain growth related parameters. Mathematical models were evaluated between modelled data and experimental data and R-square values were also computed for all the plots.

***Aureobasidium pullulans* growth:**

The lag phase of *A.pullulans* in fermentation was very short as the cells were already adapted before they were used for pullulan production. *A.pullulans* started to form pullulan instantly as the cells entered the logarithmic phase and therefore *A.pullulans* growth and pullulan production took place simultaneously. In order to validate the developed model, it is necessary to study the cell growth as function of time (i.e., Logistic growth). Sigmoidal curves are useful in describing the growth of organisms. The effect of initial substrate concentration changes on growth related parameters was done using 50, 75 and 100 g/L of jaggery and sucrose in batch fermentation for about 172 hrs. Other conditions of fermentation were kept at same values.

From experiments, maximum cell concentrations (X_m) were considered for the initial jaggery and sucrose concentrations of 50, 75 and 100 g/L, respectively. Upon (linear) fitting the experimental data in to equation (2), Logistic (L) model equation parameters, maximum specific growth rate (μ_{max}) and initial biomass concentrations (X_0) for increased concentrations of jaggery were determined. The resulting R^2 values and calculated values were summarized in Table 1. Figures 1 and 2 shows the comparison of experimental data and model predictions for *A.pullulans* growth from increasing concentrations of jaggery and sucrose, respectively.

Table 1. Kinetic parameters of Logistic (L) model for *Aureobasidium pullulans* growth on Jaggery and Sucrose

Parameters	Initial Jaggery Concentration (g/L)			Initial Sucrose Concentration (g/L)		
	50	75	100	50	75	100
μ_{max}, hr^{-1}	0.0706	0.0545	0.0679	0.048	0.0611	0.0586
R^2	0.83	0.81	0.93	0.77	0.92	0.91
X_0, g/L	2.28	0.667	0.636	4.29	0.547	1.0026
X_m, g/L	45.24	48.36	73.02	40	56	60.2

Table 2. Kinetic parameters of Logistic Incorporated Leudeking- Piret (LILP) model for Pullulan production on Jaggery and Sucrose

Parameters	Initial Jaggery Concentration (g/L)			Initial Sucrose Concentration (g/L)		
	50	75	100	50	75	100
α, g.P/g.X	0.069	0.2513	0.1895	0.0849	0.2227	0.1449
R^2	0.81	0.83	0.98	0.84	0.91	0.94
β, g.P/(g.X.hr)	0.001415	0.001158	0.00071	0.0014	0.000424	0.001329

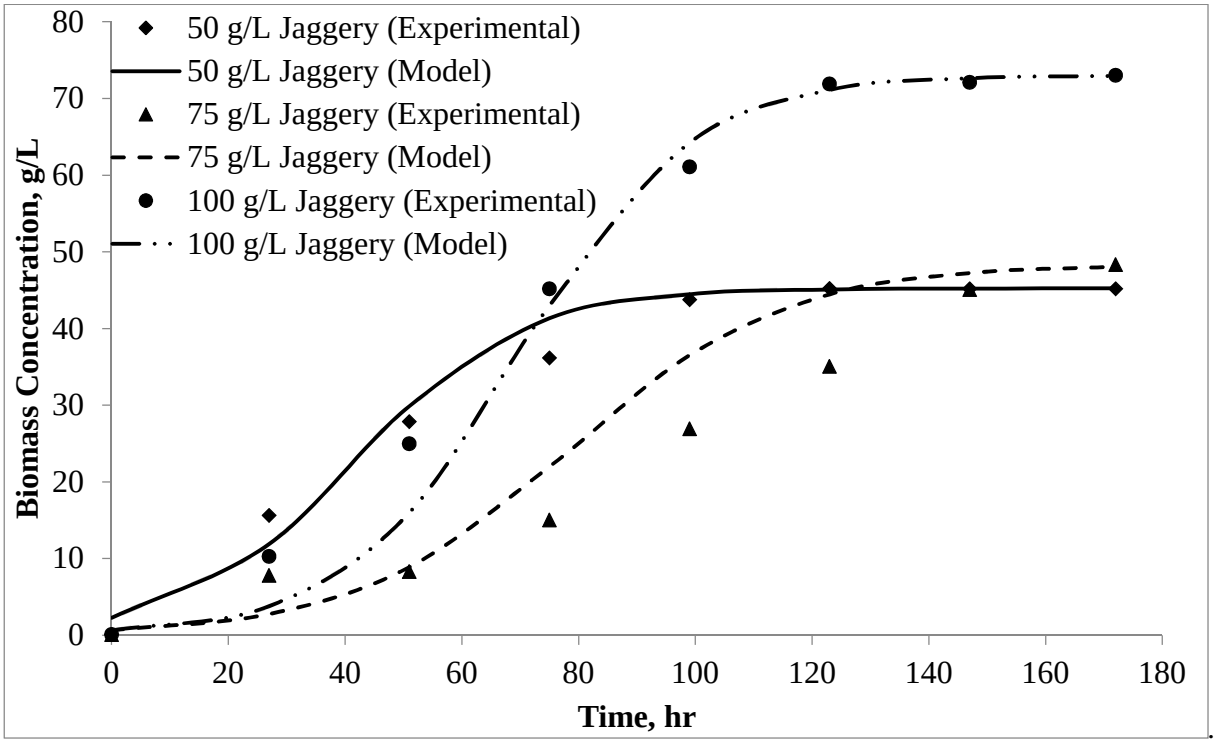

Fig.1: Growth curves of *A.pullulans* fitted with the Logistic (L) model with increasing initial concentrations (50, 75 and 100 g/L) of Jaggery as substrate

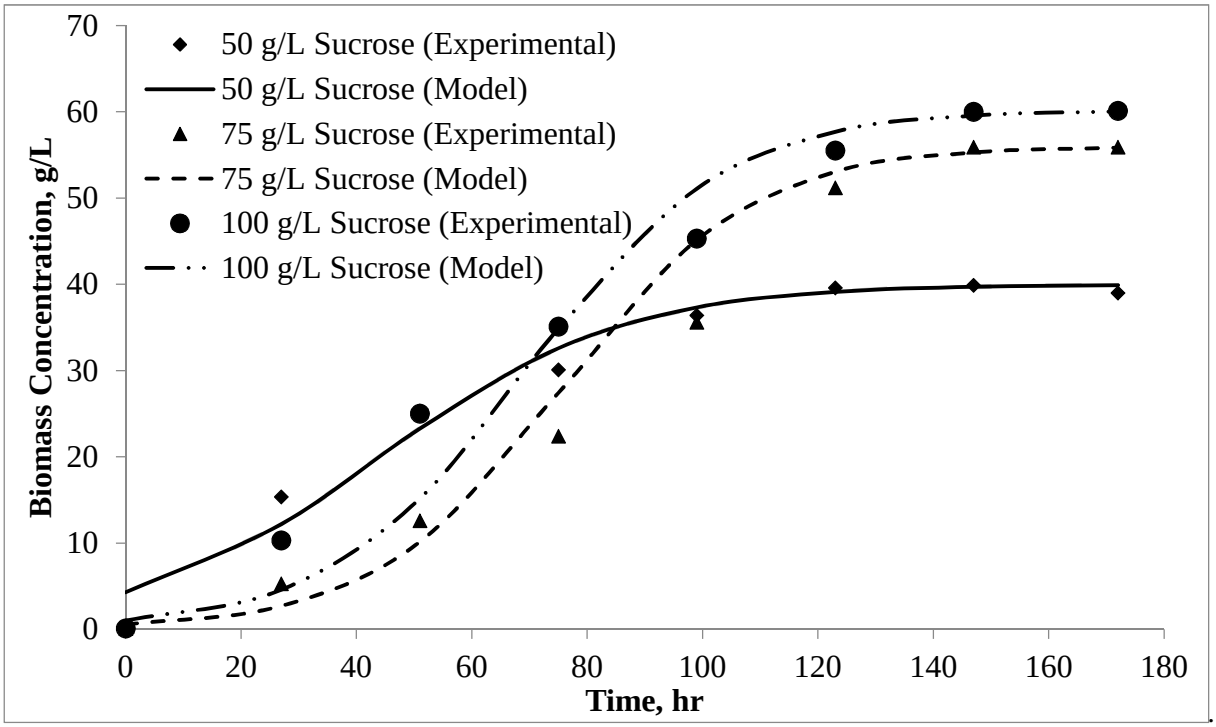

Fig.2: Growth curves of *A.pullulans* fitted with the Logistic (L) model with increasing initial concentrations (50, 75 and 100 g/L) of Sucrose as substrate

Pullulan Production

Comparisons of pullulan production profile with Logistic Incorporated Leudeking-Piret (LILP) model (equation 7) were shown by plotting both the experimental data and the predicted values from the models in Figure 3 and 4. Figure 3 represents increasing concentrations of pullulan with increasing jaggery (as the initial substrate) concentrations (50, 75 and 100 g/L). Similar curves were also observed in case of increasing sucrose (as initial

substrate) concentrations (50, 75 and 100 g/L), in Fig. 4. The LILP model did properly fit the experimental data and the estimated kinetic parameters with resulted R^2 values were listed in Table 2.

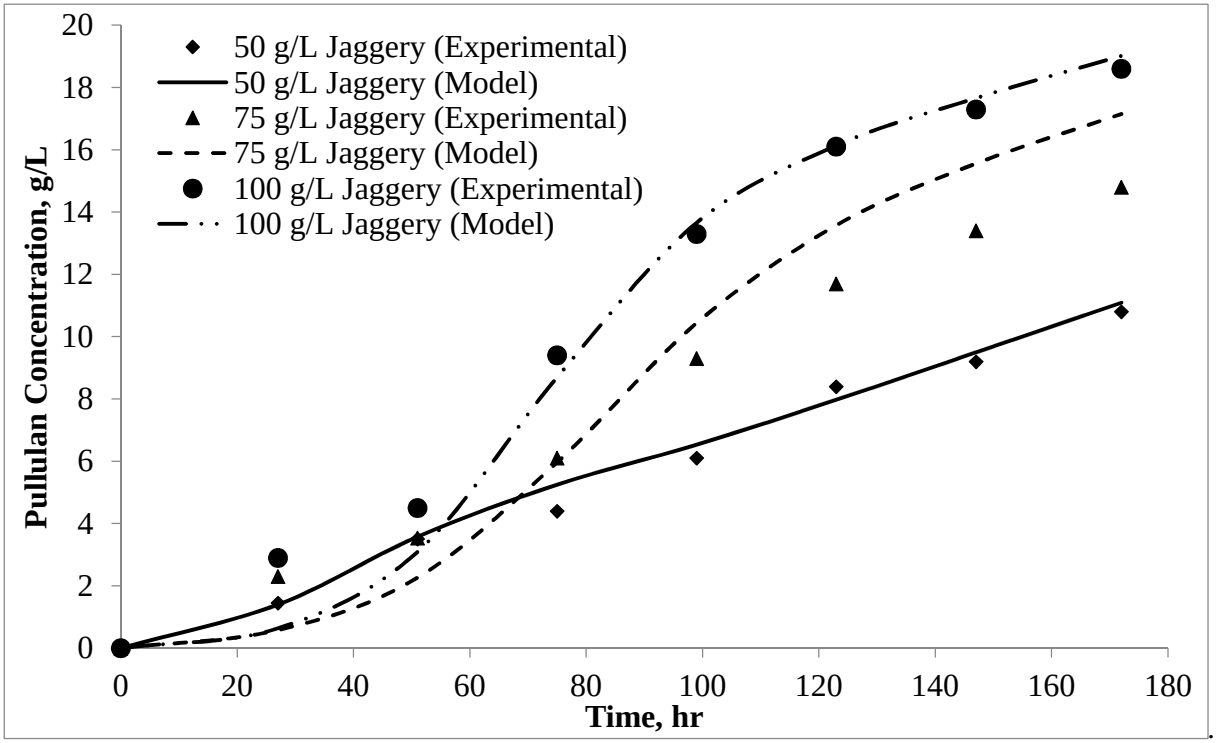

Fig.3: Profiles of produced Pullulan fitted with the Logistic Incorporated Leudeking-Piret (LILP) model with increasing initial concentrations (50, 75 and 100 g/L) of Jaggery as substrate

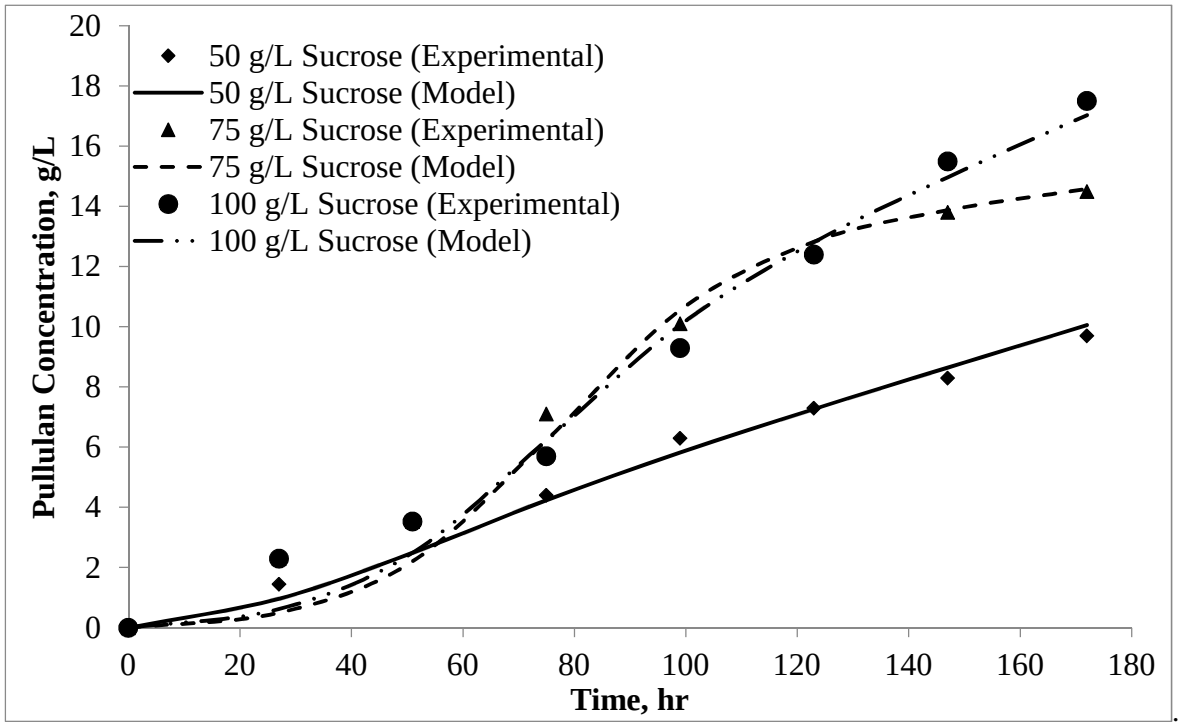

Fig.4: Profiles of produced pullulan fitted with the Logistic Incorporated Leudeking-Piret (LILP) model with increasing initial concentrations (50, 75 and 100 g/L) of Sucrose as substrate

Substrate (Jaggery and Sucrose) Consumption:

To study the substrate consumption in exopolysaccharide production, a Logistic Incorporated Modified Leudeking-Piret (LIMLP) model was applied. Subjective comparisons of actual substrate utilization by *A. pullulans* towards

pullulan production with LIMLP model were shown by plotting both experimental and predicted data from model. Figure 5 and 6 demonstrated the reasonable fit of experimental data with predicted values of model. Table 3 gives the comparison of estimated kinetic parameters of LIMLP models for initial Jaggery and sucrose concentration of 50, 75 and 100 g/L.

Table 3. Kinetic parameters of Logistic Incorporated Modified Leudeking- Piret (LIMLP) model for pullulan production on jaggery and Sucrose

Parameters	Initial jaggery concentration (g/L)			Initial sucrose concentration (g/L)		
	50	75	100	50	75	100
γ, g.*S*/g.*X*	0.8534	0.7817	1.0233	0.992	0.6138	1.2776
R^2	0.96	0.61	0.82	0.965	0.66	0.88
η, g.*S*/(g.*X*.hr)	0.00168	0.003805	0.0023	0.0019	0.0017576	0.0027907

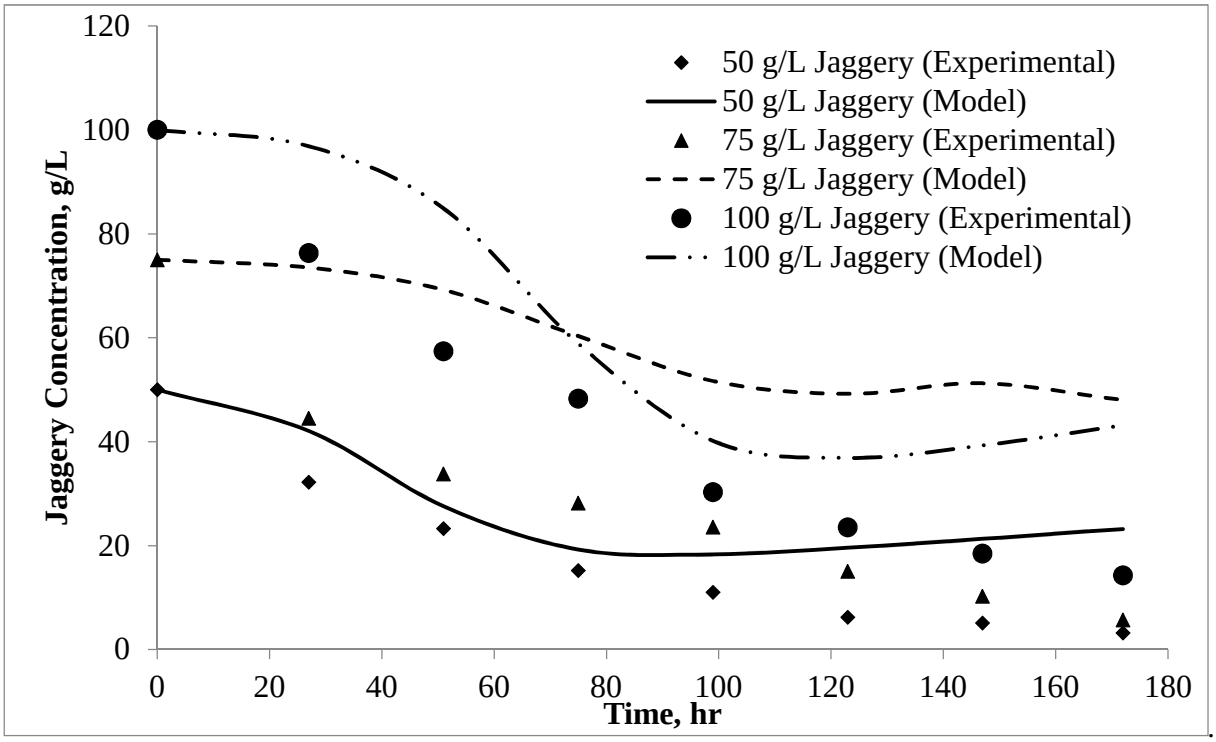

Fig.5: Profiles of jaggery (as substrate) consumption fitted with the Logistic Incorporated Modified Leudeking-Piret (LIMLP) model with increasing initial concentrations (50, 75 and 100 g/L) of jiggery

Table 4. Comparison of kinetic parameters of L, LILP and LILMP models in Pullulan fermentation using different substrates

Kinetic Parameter	K. Thirumavalavan et al., 2008 [29]	K.C.Cheng et al., 2010 [22]	Mohammad F.H.A. et al., 1995 [6]				In this study					
	Cashew Fruit Juice (g/L)	Sucrose (g/L)	Sucrose (g/L)				Jaggery (g/L)			Sucrose (g/L)		
	50	75.8	25	50	100	200	50	75	100	50	75	100
Logistic (L) Model parameters												
μ_{max}, hr^{-1}	0.07	0.048	0.035	0.042	0.002	0.023	0.0706	0.0545	0.0679	0.048	0.0611	0.0586
X_0, g/L	1.0	0.8	0.161	0.11	0.142	0.151	2.28	0.667	0.636	4.29	0.547	1.0026
X_m, g/L	92	28.3	0.501	0.792	0.923	0.721	45.24	48.36	73.02	40.0	56.0	60.2
Logistic Incorporated Leudeking-Piret (LILP) Model parameters												
α, g.*P*/g.*X*	0.9	0.79	4.75	7.69	8.89	7.14	0.069	0.2513	0.1895	0.0849	0.2227	0.1449
β, .*P*/(g.*X*.hr)	0.001	0.0047	0.0092	0.01	0.0204	0.066	0.0014	0.00116	0.00071	0.0014	0.00042	0.00133
Logistic Incorporated Modified Leudeking-Piret (LIMLP) Model parameters												
γ, g.*S*/g.*X*	0.98	2.61	3.67	3.16	4.8	5.6	0.853	0.7817	1.0233	0.992	0.6138	1.2776
η, .*S*/(g.*X*.hr)	0.001	0.007	0.008	0.0168	0.0168	0.064	0.0017	0.0038	0.0023	0.0019	0.00176	0.00279

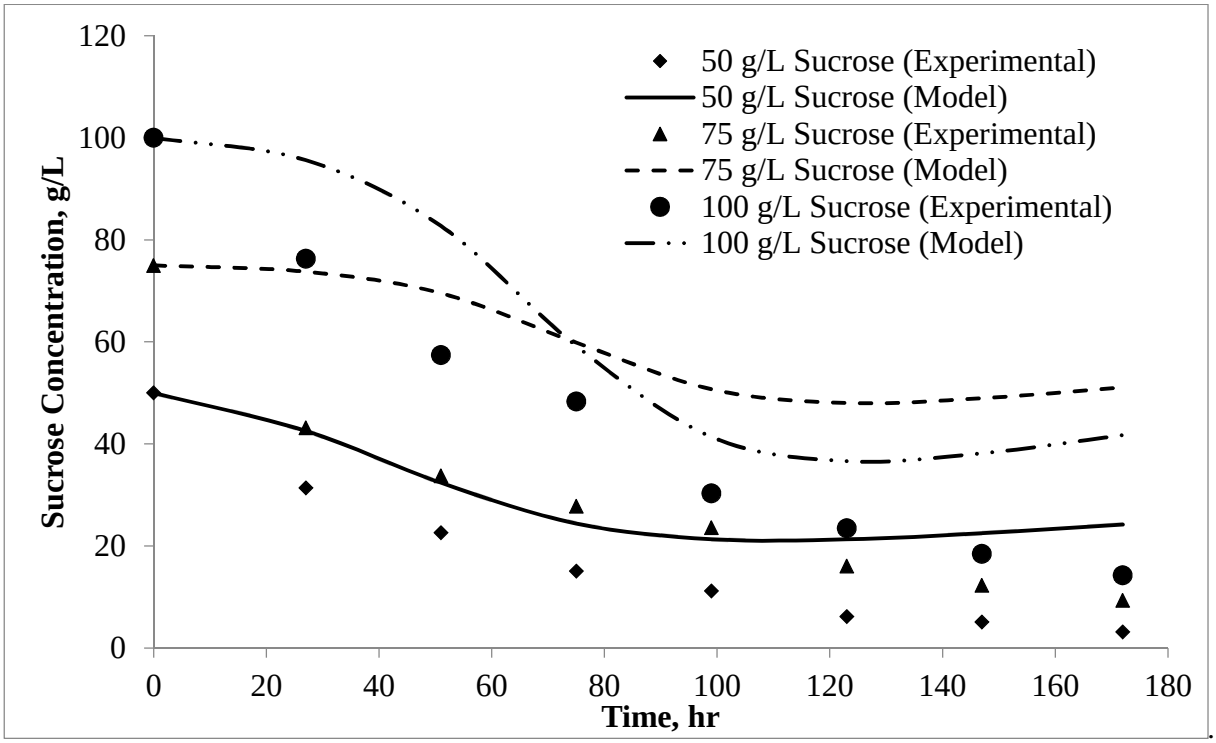

Fig.6: Profiles of sucrose (as substrate) consumption fitted with the Logistic Incorporated Modified Leudeking-Piret (LIMLP) model with increasing initial concentrations (50, 75 and 100 g/L) of sucrose

CONCLUSION

In this study, growth and non-growth related kinetic parameters, for an unstructured mathematical models of a batch shake-flask Pullulan fermentation using *Aureobasidium pullulans* using Jaggery and Sucrose (as substrate), were investigated and achieved. An increased concentrations (50, 75 and 100 g/L) of both Jaggery and sucrose were utilized for better pullulan production. Comparisons of parameters of Logistic (μ_{max}, X_0, X_m), Logistic Incorporated Leudeking-Piret (α, β) and Logistic Incorporated Modified Leudeking-Piret (γ, η) models adequately fit the experimental results with predicted data. A good concurrence of the data was shown in *A.pullulans* growth, pullulan synthesis and jaggery & sucrose utilization profiles. Estimated values of kinetic parameters were also compared with literature (Table 4). The information obtained in this study would be helpful for further developments in scaled-up productions of Pullulan.

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